PB81-100331

Cadmium Effects on the Nitrogen Fixation System of Red Alder

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1980

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1. REPORT NO.	2.	3. REC
EPA-600/J-80-035	JOURNAL ARTICLE	PB81-100331

4. TITLE AND SUBTITLE	5. REPORT DATE
Cadmium Effects on the Nitrogen Fixation of Red Alder	1980
	6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S)	8. PERFORMING ORGANIZATION REPORT NO
Carlos Wickliff, Harold J. Evans, Kevin R. Carter, and Sterling A. Russell	
9. PERFORMING ORGANIZATION NAME AND ADDRESS Environmental Research Laboratory-Corvallis Office of Research and Development U.S. Environmental Protection Agency Corvallis, Oregon 97330	10. PROGRAM ELEMENT NO
	11. CONTRACT/GRANT NO
12. SPONSORING AGENCY NAME AND ADDRESS SAME	13. TYPE OF REPORT AND PERIOD COVERED Journal article
	14. SPONSORING AGENCY CODE 600/02

15. SUPPLEMENTARY NOTES
Published in Environmental Quality, Vol. 9, No. 2, p. 180-184, 1980.

16. ABSTRACT

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When non-nodulated A. rubra seedlings were inoculated in the presence of 0.054 and 0.109 μM $CdCl_2$, N fixation decreased 52 and 89%, respectively. Plant growth decreased in a similar fashion. When non-nodulated seedlings were given 0.545 μM $CdCl_2$ and 6 mM NH_4NO_3 or $Ca(NO_3)_2$, nitrate reductase activity decreased 22 and 24%, respectively, when compared to plants receiving no $CdCl_2$. Nitrogen accretion and plant dry weights were decreased by treatments in which plants were supplied with $Ca(NO_3)_2$ and 0.273 and 0.545 μM $CdCl_2$, when compared to controls without $CdCl_2$.

These results suggest that Cd can inhibit nitrogenase activity and therefore N fixation. Growth, nodulation, and nitrate reductase activity are also inhibited by Cd.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

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NATIONAL TECHNICAL
INFORMATION SERVICE
U.S. DEPARTMENT OF COMMERCE
SPRINGFIELD, VA 22161

18. DISTRIBUTION STATEMENT	19. SECURITY CLASS (This Report)	21. NO. OF PAGES
Release to Public	Unclassified	
	20. SECURITY CLASS (This page)	22. PRICE
	Unclassified	

EPA-600/J-80-035
JOURNAL ARTICLE

Cadmium Effects on the Nitrogen Fixation System of Red Alder[1]

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ABSTRACT

Red alder (*Alnus rubra* Bong.) was grown in sand culture in the greenhouse to obtain data on the effects of cadmium (Cd) on a symbiotic nitrogen (N) fixation system which contributes to the fertility of forest soils. Treatment of red alder seedlings for 11 weeks with 0.545 to 136 μM $CdCl_2$ in N-free nutrient solution decreased in situ nitrogenase activity 25 to 89%, N fixation 23 to 98%, and the number of nodules per plant 29 to 74%, when compared to plants receiving no Cd. Similar reductions were observed in plant growth. Treatment with 0.054 to 0.545 μM $CdCl_2$ decreased nitrogenase activity 6 to 31%.

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These results suggest that Cd can inhibit nitrogenase activity and therefore N fixation. Growth, nodulation, and nitrate reductase activity are also inhibited by Cd.

Additional Index Words: **nodules, nitrogenase, acetylene reduction, $CdCl_2$ inhibition.**

Wickliff, C., H. J. Evans, K. R. Carter, and S. A. Russell. 1980. Cadmium effects on the nitrogen fixation system of red alder. J. Environ. Qual. 9:180–184.

The abundance of cadmium (Cd) from natural sources usually does not exceed 0.22 ppm. Manmade sources of environmental Cd include metallurgical and incineration processes, tire residues, and motor oils (Spec. Stud. Staff, 1975). Soil Cd has reached 1,750 ppm within 1 km of a zinc smelter (Buchauer, 1973). Roadside soil contained 1.45 ppm Cd with the concentration decreasing with increasing distance from the road, indicating that traffic was the source of the element (Lagerwerff and Specht, 1970). Cadmium concentrations in the soil of relatively unpolluted areas average 0.06 ppm and range from 0.01 to 7.0 ppm (Allaway, 1968).

Plants accumulate Cd from contaminated soils or nutrient solutions (John et al., 1972; Page et al., 1972). Cadmium increases respiration rates (Lee et al., 1976), affects mitochondrial oxidation-reduction reactions (Miller et al., 1973), and inhibits photosynthesis and nitrogen (N) fixation (Huang et al., 1974). Because these processes are critical for normal plant development, plants may be harmed by excessive Cd uptake. Growth reduction has also been observed from Cd treatment (Huang et al., 1974; Page et al., 1972). The element does not appear to be required by plants.

Alder (*Alnus* species), a known contributor of N to soils, is a hardwood tree used to make furniture and charcoal. *Alnus rubra* (Bong.), widely distributed in the Douglas fir (*Pseudotsuga menziesii*) region of the Pacific Northwest, enhances fir stand development and improves fertility of the forest soil (Tarrant, 1961; Tarrant and Miller, 1963). The mean Cd concentration in *A. rubra* from an *Alnus* stand near Yachats, Oregon, was 11 μg/g of dry plant material in both tops and roots (Wickliff and Evans, 1978). The purpose of this research was to obtain data on Cd effects on the N fixation system of *A. rubra*.

MATERIALS AND METHODS

CULTURE OF ALDER (*A. RUBRA*) SEEDLINGS

Acid-washed polyethylene containers were filled with 20-mesh silica sand which had been flushed with distilled water. Two *A. rubra* seedlings were transplanted into and cultured in each container. Seedlings were supplied with nutrient solution with or without fixed N. Excess solution drained through the polyethylene containers into receiving containers.

Alnus rubra seeds were collected from mature trees and stored 3 to 6 months at 0 to 4°C. Seeds were germinated in moist vermiculite and inoculated with a suspension of crushed *Alnus* nodules taken from roots of mature trees. Seedlings were maintained in moist vermiculite for 3 months, irrigated twice a week with nutrient solution, and then transplanted into the sand culture.

During investigation of Cd effects on plants grown with fixed N, seeds were sterilized by moderate agitation in a 5.25% solution of sodium hypochlorite (commercial bleach) for 45 min and thoroughly rinsed with sterile distilled water. These surface-sterilized seeds were germinated in autoclaved vermiculite moistened with sterile distilled water. Seedlings were given an autoclaved nutrient solution containing fixed N. After 3 months' growth, seedlings were transplanted into sand culture and supplied with nutrient solution containing fixed N. In these experiments, all containers used for culture media or other purposes were washed with 5.25% NaClO and thoroughly rinsed with sterile distilled water to prevent contamination with the *Alnus* endophyte.

Seedlings were greenhouse grown under daily supplemental fluorescent lighting of 16 hours light (minimum 5,400 lux) at 21°C and 8 hours dark at 16°C. During July and August, however, the day temperatures sometimes approached 38°C. Uniform seedlings were transplanted into sand culture and irrigated daily with 3.2 liters of nutrient solution that was collected and reused. The daily irrigation continued for 6 days; on the 7th day the used nutrient solution was discarded and the plants were irrigated with distilled water to prevent metal accumulation in the sand. Fresh nutrient solution was supplied each week.

CADMIUM TREATMENT

Before the application of Cd, plants were allowed 1 week to recover from transplanting effects. Cadmium was applied for 11 weeks to six replicate cultures of each treatment. Solutions of $CdCl_2$ from 0 to 136 μM were applied to seedlings to test Cd effects on plants fixing N. Concentrations from 0 to 0.545 μM $CdCl_2$ were used for plants supplied with fixed N.

NITROGENASE ASSAY

Nitrogenase activity was assayed in *A. rubra* nodules in situ by the acetylene reduction method (Fishbeck et al., 1973). Nodules were excised from the plant with a piece of root and assayed for 30 to 60 minutes in a serum-stoppered, 250-ml flask supplemented with 0.1 atm acetylene. There were six replicate assays per Cd treatment.

[1] Contribution from the Terrestrial Systems Div., Corvallis Environ. Res. Lab., Office of Res. and Dev., USEPA, 200 S.W. 35th Street, Corvallis, OR 97330. Received 25 June 1979.

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NITRATE REDUCTASE ASSAY

Two groups of plants were grown. One was supplied with ammonium nitrate and the other with calcium nitrate as the only source of fixed N. Cadmium solutions of 0 to 0.545 μM were applied as $CdCl_2$. After 11 weeks' growth, plants were harvested and assayed for nitrate reductase activity in roots (Li et al., 1972). Nitrate reductase activity in leaves of each plant also was assayed by the same method using 5-mm leaf discs weighing from 0.5 to 1.1 g. Six replicate assays were conducted per Cd treatment.

TOTAL NITROGEN

Plants were separated into leaves, stems, roots, and, where applicable, nodules. Total N was determined by the Kjeldahl method, using ground samples of 10 to 25 mg. A selenium modification was used for total N determination on plants grown with a fixed N source (Nelson and Sommers, 1976). The ammonia produced was measured by the alkaline phenol and sodium hypochlorite method in an auto-analyzer (Gales and Booth, 1974).

There were six replicate total N determinations per treatment in experiments with the effects of Cd on nodulated plants and on plants, without nodules, supplied with fixed N (Tables 1 and 3). In the experiment with the effect of Cd on plants nodulating after $CdCl_2$ application, the six plant samples per treatment were too small for individual N determinations. These samples were composited and N was determined in composite samples (Table 2).

TOTAL CADMIUM

The dried ground plant material remaining after the determination of the total N was used for determination of total Cd. Up to 1 g of plant material was digested 4 hours in 4 ml of concentrated HNO_3 and then a 6-ml portion of HNO_3 was added. The material was further digested with 3 ml perchloric acid until a clear digestate resulted. This digestate solution was filtered, made to 100 ml with distilled water, and analyzed for Cd by atomic absorption spectrometry. The digestate solution was compared to known concentrations of $CdCl_2$ in the perchloric acid.

In the experiment with the effect of Cd on nodulated plants, six replicate total Cd determinations were made on the leaves, stems, and roots. The six nodule samples per treatment were composited and Cd was determined in the composites because the individual samples were too small for separate determinations (Table 1). In the experiment with the effect of Cd on plants nodulating after $CdCl_2$ application, all plant samples were too small for individual Cd determinations; consequently, they were also analyzed in the latter manner (Table 2). There were six replicate Cd determinations per treatment in experiments with the effects of Cd on plants, without nodules, supplied with fixed N (Table 3).

STATISTICAL ANALYSIS

In these experiments, mean values for treatment often varied greatly with treatment standard deviations, increasing proportionally. Therefore, all data were transformed to their respective common logarithms for statistical analysis (Steel and Torrie, 1960). The tables contain a number called the *least significant percentage* (LSP) that gives the percentage increase of the larger mean over the smaller mean that is required for statistical significance.

RESULTS

CADMIUM CONTENT

In the greenhouse, Cd concentrations in the plant organs increased with increasing concentrations of the element in the nutrient solution regardless of whether

Table 1—Effect of Cd on *A. rubra* fixing N. Previously nodulated *A. rubra* plants were grown 11 weeks with the indicated concentrations of $CdCl_2$ in N-free nutrient solution.†

Conc. $CdCl_2$	Dry weight			Dry wt. per nodule	Nirogenase activity	N fixed	No. of nodules per plant‡	Cd Conc.			
	Leaves	Stems	Roots					Leaves	Stems	Roots	Nodules
μM	g			mg	μmoles ethylene formed/g of dry nodule per hour	mg/plant		μg/g of dry wt			
					Summer						
136	0.21	0.18	0.16	0.3	3.7	2.9	69	74.1	558.3	5,584.1	1,279.0
54.5	0.41	0.34	0.33	0.4	5.5	11.2	115	66.2	168.8	2,767.3	810.1
27.3	1.11	0.86	1.27	1.1	8.0	60.8	86	55.0	167.8	2,331.3	435.5
10.9	2.80	1.68	2.21	1.4	17.9	132.7	120	40.1	75.4	1,307.4	199.1
0.0	4.50	1.79	2.31	1.1	36.7	227.8	259	0.0	0.0	0.0	0.0
					Winter						
27.3	0.3	0.30	0.65	0.5	6.7	12.3	65	42.4	149.2	2,314.7	342.9
10.9	0.74	0.41	0.83	0.7	13.2	25.6	86	34.5	68.2	1,097.2	181.1
5.45	0.83	0.35	0.70	0.7	20.8	17.9	81	18.3	41.9	715.2	123.6
2.73	1.67	0.75	1.29	0.9	41.4	39.5	117	7.1	16.9	308.8	58.9
1.09	0.79	0.47	1.21	0.7	21.2	26.9	89	4.3	7.6	138.3	26.9
0.545	1.52	0.65	0.19	1.0	32.4	71.9	108	2.1	4.2	73.4	14.4
0.0	2.27	0.99	1.03	1.1	48.2	91.2	144	0.0	0.0	0.0	0.0
					Spring						
1.09	6.85	4.27	4.73	1.6	29.8	307.0	310	9.4	10.6	212.0	30.8
0.545	7.28	5.14	4.52	1.6	34.2	326.1	313	3.5	6.6	113.1	14.9
0.273	7.93	5.59	4.56	1.5	16.0	322.6	322	1.7	5.4	50.2	8.0
0.109	8.20	5.75	3.93	1.5	23.4	375.3	318	0.7	1.6	23.7	3.4
0.054	7.78	5.44	3.75	1.8	51.8	366.2	253	0.4	1.1	11.4	1.8
0.027	7.80	5.05	3.48	1.7	54.3	370.6	267	0.0	0.5	5.2	0.7
0.0	7.67	5.12	4.07	1.7	45.0	366.1	269	0.0	0.0	0.0	0.0
Summer LSP§	54	65	65	49	110	58.4	38	2.2	21	15	–
Winter LSP	55	66	42	47	74	66.4	36	5.4	7.6	54	–
Spring LSP	23	29	34	22	78	25.3	21	241	19	196	–

† All measurements were taken after harvest. Significance of differences between means was determined by the least significant difference (LSD) test (see Methods).

‡ There were 30 to 47 nodules on the roots before treatment, with $CdCl_2$.

§ LSP (least significant percentage) is the percentage increase of the larger mean over the smaller mean which is required for significance when two means are compared. Each LSP is percent of the smaller mean.

Table 2—Effect of Cd on *A. rubra* nodulating after application of $CdCl_2$. Plants were allowed 11 weeks to initiate nodulation with the indicated concentrations of $CdCl_2$ in the nutrient solution.†

Conc. $CdCl_2$	Dry weight				N fixed	No. of nodules per plant	Growth period prior to nodulation	Cd conc.			
	Leaves	Stems	Roots	Nodules				Leaves	Stems	Roots	Nodules
μM	mg				mg/plant		weeks	μg/g dry wt			
0.545	22	8	10	2	0	3	8	2.9	3.7	119.9	42.6
0.273	42	11	17	3	0	7	6.5	1.9	2.0	50.5	14.4
0.109	54	14	17	5	0.5	11	6	0.8	1.6	18.6	4.7
0.054	61	22	32	7	1.8	17	5	0.4	0.9	9.0	4.5
0.0	164	39	52	13	3.7	31	4.5	0.0	0.0	0.0	0.0
LSP‡	261	135	128	180	–	206	–	–	–	–	–

† All measurements, except growth period prior to nodulation, were taken after harvest. Significance of differences between means of the dry weights and numbers of nodules per plant was determined using the least significant difference test (see Methods). The N and Cd determinations were made on composite samples of all six replicates per treatment; therefore, no LSP was calculated.

‡ LSP (least significant percentage) is the percent increase of the larger mean over the smaller mean which is required for significance when two means are compared. Each LSP is percent of the smaller mean.

Table 3—Effect of Cd on *A. rubra* supplied with fixed N. Plants were grown without nodules, for 11 weeks in nutrient solution containing 6 mM NH_4NO_3 or $Ca(NO_3)_2$ and the indicated concentrations of $CdCl_2$.†

Source of fixed N	Conc. $CdCl_2$	Dry weight			Nitrate reductase activity		N accretion	Cd conc.		
		Leaves	Stems	Roots	Leaves	Roots		Leaves	Stems	Roots
	μM	mg			μmoles NO_3^- formed/g dry wt per hour		MG/plant	μg/g dry wt		
NH_4NO_3	0.545	598	311	368	1.81	2.50	22	2.9	3.6	165
	0.273	557	323	405	2.20	2.95	22	2.1	2.0	81
	0.109	894	513	637	2.23	3.07	37	1.6	0.9	32
	0.0	923	511	833	2.50	3.06	41	0.0	0.0	0
$Ca(NO_3)_2$	0.545	143	93	99	1.79	1.86	2.8	3.0	3.5	167
	0.273	231	133	142	2.06	2.41	5.7	2.2	1.8	71
	0.109	318	157	200	2.34	2.66	9.0	1.6	0.9	31
	0.0	507	274	393	2.14	2.55	14.7	0.0	0.0	0
NH_4NO_3	LSP‡	59	53	109	32	37	86	64	87	43
$Ca(NO_3)_2$	LSP‡	87	59	97	23	27	138	47	40	42

† All measurements were taken after harvest. Significance of difference between means was determined using the least significant difference test (see Methods).

‡ LSP (least significant percentage) is the percent increase of the larger mean over the smaller mean which is required for significance when two means are compared. Each LSP is percent of the smaller mean.

the plants were fixing atmospheric N or were supplied with fixed N (Tables 1–3). The concentrations in the roots were 4 to 8, 9 to 20, and 30 to 75 times those in the nodules, stems, and leaves, respectively.

EFFECT OF CADMIUM ON NODULATED PLANTS

The dry weight of *A. rubra* organs decreased as Cd increased in the range of 10.9 to 136 μM $CdCl_2$ in the nutrient solution (Table 1). The greatest reductions in dry weight occurred between 10.9 and 54.5 μM. Growth reduction was less evident in experiments using 0.545 to 27.3 μM $CdCl_2$ and was not seen when 0.027 to 0.545 μM $CdCl_2$ was applied. The dry weights were significantly reduced in leaves of plants given 1.09 to 136 μM $CdCl_2$ (Table 1).

Nitrogenase-dependent acetylene reduction rate decreased in nodules as the $CdCl_2$ concentration increased (Table 1). Although an apparent increase in nitrogenase activity occurred at 2.37 μM $CdCl_2$, this effect was not observed in later experiments.

The amount of N fixed by *A. rubra* decreased as Cd concentration increased from 1.09 to 136 μM $CdCl_2$ in the nutrient solution (Table 1). Nitrogen fixation did not decrease significantly from 0.027 to 0.545 μM $CdCl_2$.

Fewer root nodules were found following growth on nutrient solution containing from 10.9 to 136 μM $CdCl_2$ than on Cd-free nutrient solution (Table 1). The dry weight increase per nodule was less at 54.5 and 136 μM $CdCl_2$ than at 0 μM.

EFFECT OF CADMIUM ON *A. RUBRA* NODULATING AFTER APPLICATION OF $CdCl_2$

Plants without nodules that were inoculated when Cd was added to the nutrient solution grew more slowly than those with nodules prior to addition of Cd. Cadmium effects on dry weights of plant organs were greatest at the highest Cd concentration (Table 2). Each plant had fewer nodules and the nodule dry weights increased less as Cd increased from 0 to 0.545 μM $CdCl_2$. Dark green leaves indicated that nodulation had occurred and the plants were receiving sufficient N for growth. Plants without nodules did not grow. The time after inoculation necessary for nodulation increased as Cd concentration increased (Table 2). These results indicate that Cd inhibited the initiation of nodulation.

Nitrogenase-dependent acetylene reduction was unchanged following treatment of *A. rubra* with from 0.054 to 0.545 μM $CdCl_2$ in the nutrient solution (data not presented). Nitrogen fixation decreased as Cd con-

centration increased (Table 2). No N fixation was detected at 0.273 and 0.545 μM $CdCl_2$.

Cadmium concentration increased in the organs of *A. rubra* plants that were allowed to initiate nodulation with 0 to 0.545 μM $Cdcl_2$ in the nutrient solution (Table 2). The roots accumulated more Cd than the leaves, stems, and nodules.

EFFECT OF CADMIUM ON PLANTS SUPPLIED WITH FIXED NITROGEN

Alnus rubra plants were cultured in nutrient solution containing 6 mM nitrogen as either NH_4NO_3 or $Ca(NO_3)_2$. Plants supplied with NH_4NO_3 were larger than those that received $Ca(NO_3)_2$ (Table 13). There were no root nodules in either group of plants. Dry matter accumulation in the plant organs decreased significantly with increasing Cd concentration when $Ca(NO_3)_2$ was the N source. In addition, significant decreases occurred in dry matter accumulation in plants given 0.273 and 0.545 μM $CdCl_2$ in nutrient solutions containing NH_4NO_3.

The nitrate reductase activities in leaves and roots of plants given 0.545 μM $CdCl_2$ were significantly decreased when NH_4NO_3 or $Ca(NO_3)_2$ was the nitrogen source (Table 3). Nitrogen accretion during growth was significantly decreased at all Cd concentrations with $Ca(NO_3)_2$ as the N source. Decreases occurred at 0.273 and 0.545 μm $CdCl_2$ when NH_4NO_3 was the N source.

Cadmium concentrations in the plant organs increased as the concentration of $CdCl_2$ in the nutrient solution increased. Roots accumulated more Cd than leaves and stems.

DISCUSSION

These experiments investigated the Cd effects on the N fixation system of red alder (*Alnus rubra*). Plant responses to Cd treatment were similar to those reported by previous investigators (Huang et al., 1977; John et al., 1972; Page et al., 1972). Responses included growth reduction of all organs, inhibition or destruction of nitrogenase activity, and accumulation of Cd in the organs.

Alnus rubra grew very slowly at 136 μM $CdCl_2$. Chlorosis of the leaves indicated that chlorophyll biosynthesis was impaired. Photosynthetic rates and therefore available carbohydrate probably decreased. Because ATP is required for nitrogenase activity and is provided by oxidative phosphorylation in the cells of the endophyte (van Straten et al., 1977) decreased availability of carbohydrate is expected to limit N fixation in the nodules of *A. rubra* and other N-fixing species.

Cadmium was translocated from roots to the leaves. Thus, the element may inhibit photosynthesis by reacting the plant proteins and reducing the activity of enzymes involved in photosynthetic reactions. Cadmium was clearly detrimental to the N fixation system of *A. rubra* with 0.545 to 136 μM $CdCl_2$ in the nutrient solution.

Cadmium chloride at 0.027 to 1.09 μM in the nutrient solution did not significantly alter the growth and N accumulation of *A. rubra*. Nitrogenase activity, however, was decreased in the range of 0.109 to 1.09 μM $CdCl_2$ (Table 1). Further nodulation of previously nodulated plants increased with increasing Cd in this concentration range. Reduced enzyme activity was apparently compensated for by formation of more nodules on the roots.

Three ranges of Cd concentration (10.9 to 136 μM, 0.545 to 27.3 μM, and 0.037 to 1.09 μM) were given to plants at different times of the year (Table 1). The most important environmental variables with respect to time of year are light intensity, temperature (and concomitant relative humidity), and moisture variations of growth medium, all of which affect plant growth. Thus, Cd effects on plant growth at one time of the year may not be comparable with effects during another growth period.

Water or dilute acid-extractable Cd varied little in the culture medium. The Cd concentrations varied little in plants grown at the different time periods. These results indicate that Cd availability to plants and uptake of the element by roots were unaltered by the environmental variables discussed.

Plants cultured in nutrient solution containing 6 mM NH_4NO_3 were about twice the size of those given 6 mM $Ca(NO_3)_2$ (Table 3). The different responses to N forms complicated the experiment and obscured Cd effects on growth. It was shown, however, that Cd inhibited nitrate reductase activity and growth of non-nodulated *A. rubra* when nitrate in growth-limiting concentrations is used as a source of fixed N.

These results support earlier observations that Cd inhibits N fixation (Huang et al., 1974). Nitrogenase activity within the nodules was inhibited when the nodules contained at least 3.4 μg Cd/g dry matter. The N fixation system of *A. rubra* may be inhibited in contaminated forests that allow this or a greater concentration of Cd to occur in the nodules.

ACKNOWLEDGMENTS

We wish to thank Drs. Donald B. Zobel, David P. Moore, and Ralph S. Quatrano for their helpful suggestions in the design of this investigation. We also thank Mr. Louis Grothaus for suggesting the statistical procedures used in this report and Nancy Lanpheare for preparing the manuscript.

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